PREPARATION AND PROPERTIES OF DERIVATIVES OF VIRGINIAMYCIN S

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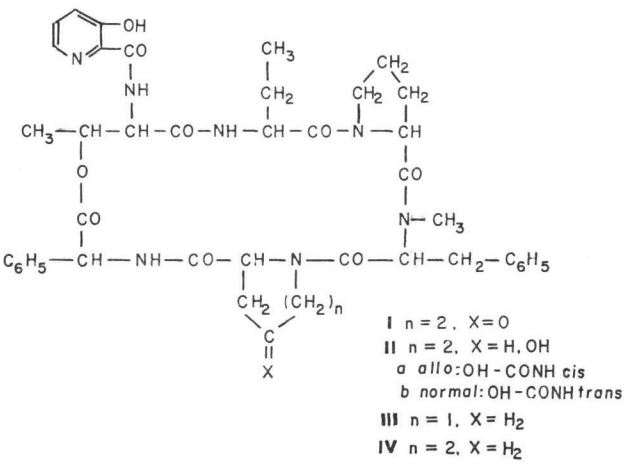
Reduction of virginiamycin S with sodium borohydride produces *allo*- and *normal*-dihydrovirginiamycin S. Reduction of the tosylhydrazone of virginiamycin S with sodium cyanoborohydride affords deoxyvirginiamycin S. These compounds are less active than virginiamycin S. Like virginiamycin S they enhance the activity of virginiamycin M_1.

The antibiotics streptogramin[1], PA–114[2], ostreogrycin[3], virginiamycin[4,5], mikamycin[6], vernamycin[7] and pristinamycin[8] are mixtures of two types of peptolides, *i.e.* a macrocyclic lactone containing a peptide part. With each antibiotic the compounds of both types exhibit a mutual synergism. No chemical modifications have been described for the peptolides containing a large hydrocarbon moiety (*e.g.* ostreogrycin A, virginiamycin M). Chemical modifications of the peptolides consisting mainly of amino acids, also called cyclodepsipeptides, have been limited to the preparation of dihydrovirginiamycin S (**II**) by reduction of virginiamycin S (**I**)[9]. The total synthesis of a related cyclodepsipeptide, patricin A (**III**) has been described[10,11]. This compound structurally corresponds to virginiamycin S in which 4-oxo-pipecolic acid is replaced by proline. Dihydrovirginiamycin S[9] and patricin A[10] enhance the antibacterial activity of virginiamycin M_1 and of vernamycin A; these two last compounds are in fact identical[12].

In order to study the influence of structural modifications on the antibacterial activity, we re-examined the preparation of dihydrovirginiamycin S (**II**) and we prepared deoxyvirginiamycin S (**IV**). We also prepared tritium labeled dihydrovirginiamycin S for some studies of the mechanism of action of this antibiotic[13].

Treatment of virginiamycin S with sodium borohydride reduces the carbonyl group of the 4-oxo-pipecolic acid moiety yielding both epimeric hydroxy derivatives. These dihydro compounds (**IIa** and **IIb**) can be separated by thin-layer chromatography and by preparative column chromatography. Their structures were confirmed by mass spectrometry[14]. The configuration of the hydroxyl groups in these dihydrovirginiamycins S was determined by identification of the 4-

Fig. 1. Structure of virginiamycin S and derivatives.



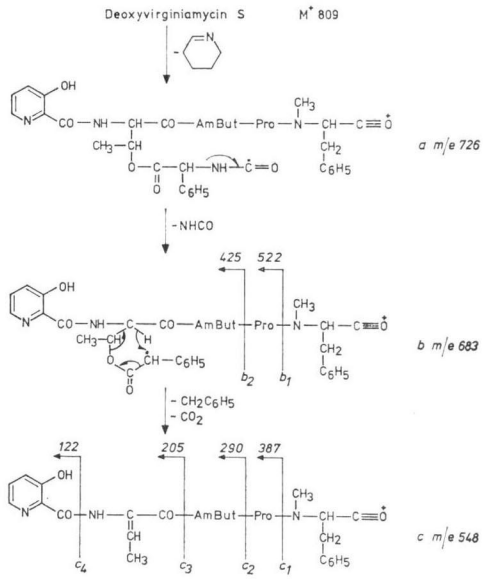
hydroxypipecolic acids formed by acid hydrolysis[15]. Two-dimensional paper chromatography of the hydrolysates in *n*-butanol - acetic acid - water (4: 1: 5) followed by *tert*-amyl alcohol - 2,4-lutidine - water (178: 178: 114) and comparison with authentic samples showed that the dihydro derivatives **IIa** and **IIb** contain 4-*allo*-(OH–COOH *cis*) and 4-*normal*-(OH–COOH *trans*) hydroxypipecolic acids, respectively.

The reduction of virginiamycin S with sodium borohydride produces the *allo*- and *normal*-dihydro derivatives in a 2: 1 ratio. 4-*Allo*-Hydroxypipecolic acid bears an equatorial hydroxyl group. Predominant formation of the equatorial orientation has often been observed upon reduction of cyclic ketones[16].

The reduction of virginiamycin S with tritiated sodium borohydride proceeds in the same way giving *allo*- and *normal*-dihydrovirginiamycin S-^{3}H with a specific activity of 27.9 and 19.4 mCi/mmole, respectively.

The deoxygenation of virginiamycin S was performed by reduction of the tosylhydrazone with sodium cyanoborohydride. As reported these reductions give good yields by heating the tosylhydrazone with sodium cyanoborohydride and *p*-toluenesulfonic acid at 100~110°C in a 1: 1 mixture of dimethylformamide and sulfolane[17]. Under these conditions some deoxyvirginiamycin S is obtained but the presence of these solvents hinders the isolation of the reaction products. We found that the reduction is more favorably carried out in methanol at room temperature; the yield is probably slightly lower but the isolation is easier. In dimethylformamide or dioxane only decomposition products are formed.

Fig. 2. Azomethin degradation pathway of deoxyvirginiamycin S.



Mass spectrometry confirmed the structure of deoxyvirginiamycin S. The mass spectrum shows an intense molecular ion at *m/e* 809 and a fragmentation pattern similar to that of virginiamycin S[14]. The predominant azomethin degradation pathway starts with elimination of pipecolic acid as 1,2-dehydropiperidine yielding the fragment ions: M → a → b (→ b_1 → b_2) → c → c_1 → c_2 → c_3 → c_4 (Fig. 2). The presence of pipecolic acid is further demonstrated by an intense fragment at *m/e* 84 representing the 1,2-dehydropiperidinium ion.

The synthesis of deoxyvirginiamycin S, called patricin B, according to the scheme used for the preparation of patricin A, has been mentioned[11]. No physico-chemical or biological properties were reported.

Both dihydrovirginiamycins S and deoxyvirginiamycin S are less active than virginiamycin S. The minimum inhibitory concentrations of these compounds and of patricin A against *Staphylococcus aureus, Bacillus subtilis* and *B. cereus* are listed in Table 1. The compounds, like virginiamycin S[5], enhance the antibacterial activity of virginiamycin M_1. The synergistic effects against *S. aureus* and *B. subtilis* are reported in Tables 2 and 3, respectively.

Table 1. Minimum inhibitory concentrations (μg/ml) of virginiamycin S and related compounds.

	Virginiamycin				Patricin A
	S	dihydro S		deoxy S	
		allo	*normal*		
Staph. aureus ATCC 6538P	12.50	50	50	50	50
B. subtilis NCTC 8236	3.12	12.50	12.50	6.25	100
B. cereus NCTC 8035	1.56	50	50	50	100

Table 2. Minimum inhibitory concentrations (μg/ml) of mixtures of virginiamycin M_1 with virginiamycin S and related compounds against *Staphylococcus aureus* ATCC 6538P.

% M_1	% S component	Virginiamycin				Patricin A
		S	dihydro S		deoxy S	
			allo	*normal*		
100	—	0.391				
90	10	0.024	0.098	0.049	0.098	0.195
70	30	0.024	0.049	0.049	0.049	0.195
50	50	0.024	0.049	0.049	0.049	0.195

Table 3. Minimum inhibitory concentrations (μg/ml) of mixtures of virginiamycin M_1 with virginiamycin S and related compounds against *B. subtilis* NCTC 8236.

% M_1	% S component	Virginiamycin				Patricin A
		S	dihydro S		deoxy S	
			allo	*normal*		
100	—	0.781				
90	10	0.195	0.098	0.195	0.098	0.195
70	30	0.098	0.098	0.098	0.024	0.098
50	50	0.049	0.049	0.098	0.024	0.098

Experimental

Allo- and *normal*-Dihydrovirginiamycin S (**IIa** and **IIb**)

To a solution of 250 mg of virginiamycin S in 25 ml of ethanol was added 12.5 mg of sodium borohydride dissolved in 5 ml of water. The pH of the solution was adjusted to 8.2~8.5 by addition of a 5% acetic acid solution. The reaction mixture was kept at room temperature for 1 hour and was then acidified to pH 4 with dilute hydrochloric acid followed by neutralization with dilute sodium hydroxide. Ethanol was removed under reduced pressure. The precipitated dihydrovirginiamycin S was extracted with a mixture of ether - methylene chloride (5: 1) and this solution was washed with water, dried and evaporated. Thin-layer chromatography of the residue on silica gel (precoated plates of silica gel 60, Merck) with chloroform - methanol (95: 5) and detection at 360 nm revealed the presence of two products, **IIa** and **IIb**, with Rf's of 0.51 and 0.40, respectively (Rf of virginiamycin S 0.64). The residue (220 mg) was dissolved in chloroform and was chromatographed on a column of 12.5 g of silica gel (finer than 230 mesh ASTM). Elution with chloroform - methanol (99: 1) afforded 100 mg of *allo*-dihydrovirginiamycin S (**IIa**). Recrystallization of this compound from methanol gave a product which became transparent at 159~160°C and liquefied at about 170°C, UV maxima in ethanol, 306 nm (log ε 3.88) and 352 nm (log ε 2.85).

Anal. Calcd. for $C_{43}H_{51}N_7O_{10}$ (825.85): C, 62.53; H, 6.22; N, 11.87.
Found: C, 62.40; H, 6.35; N, 11.99.

Further elution afforded 60 mg of the normal epimer (**IIb**), which was recrystallized from methanol, mp 235~238°C, UV maxima in ethanol, 307 nm (log ε 3.85) and 353 nm (log ε 2.83).

Anal. Calcd. for $C_{43}H_{51}N_7O_{10}$ (825.85): C, 62.53; H, 6.22; N, 11.87.
Found: C, 62.36; H, 6.34; N, 12.05.

Hydrolysis of Dihydrovirginiamycin S

Allo- and *normal*-dihydrovirginiamycin S (**IIa** and **IIb**) were hydrolyzed separately in sealed tubes with 6 N hydrochloric acid (10 mg/ml) at 100°C for 24 hours. Following evaporation, the residue was repeatedly dissolved in water and evaporated to remove residual hydrochloric acid. Paper chromatography of the residue on Whatman No. 1 with *n*-butanol - acetic acid - water (4: 1: 5) and detection with ninhydrin reagent showed the presence of threonine (Rf 0.20), 4-*allo*-hydroxypipecolic acid (OH–COOH *cis;* Rf 0.19), 4-*normal*-hydroxypipecolic acid (OH–COOH *trans*; Rf 0.21), proline (Rf 0.30), 2-aminobutyric acid (Rf 0.35), phenylglycine (Rf 0.46) and N-methyl-phenylalanine (Rf 0.60). Coloration with ferric chloride showed 3-hydroxypicolinic acid (Rf 0.50).

The 4-*allo-* and 4-*normal*-hydroxypipecolic acids were identified by two-dimensional paper chromatography of the hydrolysates on Whatman No. 1 in *n*-butanol - acetic acid - water (4: 1: 5) followed by *tert*-amyl alcohol - 2,4-lutidine - water (178: 178: 114)[15]. The hydrolysate of **IIa** contained 4-*allo*-hydroxypipecolic acid and that of **IIb** 4-*normal*-hydroxypipecolic acid, with Rf's relative to alanine in the *tert*-amyl alcohol - 2,4-lutidine - water solvent of 0.93 and 1.35, respectively (Rf relative to alanine of threonine 1.10).

Dihydrovirginiamycin S-^{3}H

The reduction of virginiamycin S (100 mg) with tritiated sodium borohydride (6 mg, New England Nuclear Corp.) with a specific activity of 200 mCi/mmole was carried out as described above for the sodium borohydride reduction. Any labile tritium was removed by repeated equilibrations of the reaction product in methanol. Separation of both epimers on a column of silica gel afforded 52 mg of *allo*-dihydrovirginiamycin S-^{3}H with a specific activity of 27.9 mCi/mmole and 15 mg of *normal*-dihydrovirginiamycin S-^{3}H with a specific activity of 19.4 mCi/mmole.

Radio-activity was measured with a liquid scintillation counter (Nuclear Chicago). A solution (50 μl) of the radio-active compound in methanol (100 μg/ml) was added to the scintillation liquid (4 g of 2,5-diphenyloxazole and 0.1 g of 2,2′-*p*-phenylbis-5-phenyloxazole in 1,000 ml of toluene). Counting was carried out at 0°C for 10 minutes.

Deoxyvirginiamycin S (**IV**)

A solution of 500 mg of virginiamycin S and 140 mg of *p*-toluenesulfonylhydrazine in 6 ml of ethanol was refluxed for 30 minutes. The solvent was evaporated, the residue dissolved in 30 ml of methanol containing 300 mg of *p*-toluenesulfonic acid monohydrate and to this solution 1.5 g of sodium cyanoborohydride was added. The resulting solution was kept under nitrogen for 16 hours at room temperature. The reaction mixture was then diluted with 10 ml of water and neutralized with 1.0 N hydrochloric acid. Methanol was evaporated under reduced pressure and the precipitate formed was extracted with methylene chloride. The methylene chloride solution was washed with water, dried and evaporated. The residue was chromatographed on 40 g of silica gel (70~230 mesh ASTM). Elution with chloroform - methanol (99.5: 0.5) afforded 120 mg of pure material. Recrystallization from methanol gave 75 mg of deoxyvirginiamycin S, which shows an indefinite melting point from 137° to 150°C. Its purity was checked by thin-layer chromatography on silica gel (precoated plates of silica gel 60, Merck) with chloroform - methanol (96: 4) and detection with 360 nm UV-light, Rf 0.59 (Rf of virginiamycin S 0.50), UV maxima in ethanol, 306 nm (log ε 4.17) and 364 nm (log ε 2.91).

The mass spectrum of deoxyvirginiamycin S was obtained with an AEI–MS12 mass spectrometer, accelerating voltage 8 kV, trap current 500 μA, ionization energy 70 eV, source temperature 225°C. The sample was introduced with the direct insertion probe. Diagnostic fragment ions are: *m/e* (relative intensity) (the explanation of the letters is in Fig. 2): 809 (27.1) M; 781 (3.1) M–CO; 765 (0.3) M−CO_2; 726 (3.6) a; 718 (1.5) M−$C_6H_5CH_2$; 683 (37.8) b; 676 (1.9) M−C_6H_5CH=NH, −CO; 660 (1.2) M−C_6H_5CH=NH, −CO_2; 648 (2.7) M−C_6H_5CHCO, −HNCO; 620 (0.7) M−$C_6H_5CH_2CHCO$, −CH_3NCO; 548 (1.3) c; 522 (5.3) b_1; 425 (4.0) b_2; 387 (2.0) c_1; 290 (25.3) c_2; 205 (44.4) c_3; 134 (32.4)

$C_6H_5CH_2CH=\overset{+}{N}HCH_3$; 122 (57.8) c_4; 106 (30.7) $C_6H_5CH=\overset{+}{N}H_2$; 84 (95.6) $\overset{+}{N}H=CH(CH_2)_4$; 70 (100) $\overset{+}{N}H=CH(CH_2)_3$.

Antibacterial Activity

Solutions of the compounds in ethanol (1 mg/ml) were serially (1/2) diluted to 0.03 μg/ml final concentration with 0.9% NaCl solution and each of these solutions was further diluted 1/10 with the medium. The minimum inhibitory concentrations (MIC) were determined by streak inoculation with the microorganism and incubation at 37°C for 20 hours.

For the synergism studies ethanolic solutions of the compounds (1 mg/ml) were diluted 1/5 with 0.9% NaCl solution and mixtures were made from appropriate amounts of these solutions. The resulting solutions were serially (1/2) diluted to 0.003 μg/ml final concentration with Trypticase Soy broth containing phenol red as indicator and inoculated with the microorganism. The MICs were determined after incubation at 37°C for 20 hours.

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